

The conserved *Schizosaccharomyces pombe* kinase *plp1*, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G₁ and G₂ cells

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We have identified a *Schizosaccharomyces pombe* gene with homology to the budding yeast gene *CDC5*, the *Drosophila* gene *polo*, and the mammalian family of genes encoding polo-like kinases. Disruption of this gene, *plp1*⁺, indicates that it is essential. Loss of *plp1*⁺ function leads to a mitotic arrest in which condensed chromosomes are associated with a monopolar spindle or to the failure of septation following the completion of nuclear division. In the latter case, cells show a failure both in the formation of an F-actin ring and in the deposition of septal material, suggesting that *plp1*⁺ function is required high in the regulatory cascade that controls septation. The overexpression of *plp1*⁺ in wild-type cells also results in the formation of monopolar spindles but also induces the formation of multiple septa without nuclear division. Septation can also be induced in the absence of mitotic commitment and concomitant spindle formation by the overexpression of *plp1*⁺ in *cdc25-22* or *cdc2-33* cells arrested in G₂; in G₁ cells arrested at Start by the *cdc10-V50* mutation, or in cells lacking the cyclin B homolog *cdc13* that undergo repeated S phases in the absence of mitosis.

[Key Words: *S. pombe*; polo homolog; bipolar spindle; actin ring; septum; *plp1* kinase]

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The *polo*¹ mutation of *Drosophila* was first identified as a maternal-effect mutant giving rise to embryos that show abnormal networks of microtubules associated with condensed chromosomes, in which the centrosomal antigen CP190 (formerly called Bx63; Frasch et al. 1986; Whitfield et al. 1988) fails to become organized into centrosomes with microtubule-organizing capability (Sunkel and Glover 1988). The mutation results in multipolar spindles and nondisjunction in male meiosis leading to reduced fertility. There is a high mitotic index in larval neuroblasts, with a wide range of defects including monopolar spindles and spindles with broad poles (Sunkel and Glover 1988; Llamazares et al. 1991).

polo encodes a 576-amino-acid protein that has an amino-terminal putative kinase domain and a 300-residue carboxy-terminal domain (Llamazares et al. 1991). The *polo* kinase is highly conserved. The budding yeast and murine genes, *CDC5* (Kitada et al. 1993) and mouse *Plk* (polo-like kinase; Clay et al. 1993; Lake and Jelinek 1993; Hamanaka et al. 1994), for example, show 52% and 65% identity in the kinase domain and 33% and 43% in the carboxy-terminal domain, with the latter homolo-

gies lying in distinct blocks. The human Plk has also been identified (Golsteyn et al. 1994; Hamanaka et al. 1994; Holtrich et al. 1994), and studies in the mouse suggest that there is another closely related kinase, Snk (serum-induced kinase; Simmons et al. 1992), that falls into the same gene family. These mammalian genes are expressed only in proliferating cells or tissues suggesting a cell cycle role.

In mutants of the budding yeast gene *CDC5*, nuclear division is arrested at a late stage and the spindle is elongated (Hartwell et al. 1973; Byers and Goetsch 1974). Furthermore, when temperature-sensitive *cdc5* mutants are shifted to restrictive temperature after initiating the first meiotic division, two spindles form that do not elongate and the four poles encapsulate into two diploid spores (Schild and Byers 1980). Although the activity of *CDC5* kinase has not been determined with respect to cell cycle progression, its transcript is periodically accumulated around G₂/M (Kitada et al. 1993). Some circumstantial observations suggest that *polo/CDC5* may regulate microtubule behavior. *cdc5* mutants show an unusual interaction with MBC, a drug that binds tubulin and depolymerizes microtubules (Wood and Hartwell 1982), and there is a strong interaction of *polo* with mu-

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tations in *asp* that appear to affect microtubule stability (C. Gonzalez, C.E. Sunkel, and D.M. Glover, unpubl.).

The mitotic stage at which polo kinase appears maximally active has only been determined in the syncytial embryos of *Drosophila* where the activity peaks at late anaphase–telophase (Fenton and Glover 1993). This would be consistent with a role late in the mitotic cycle as suggested by the phenotype of the extant *cdc5* mutants.

In this paper we report the isolation of a gene homologous to *polo* from *Schizosaccharomyces pombe* and describe the phenotypes that result from its loss of function or overexpression. These observations point to a key role for the enzyme not only in establishing a bipolar spindle but also in promoting septation.

Results

Conserved elements lie in both catalytic and carboxy-terminal noncatalytic domains of *S. pombe* *plol*⁺

The high degree of conservation extending from the catalytic domain into the carboxy-terminal domain of the *Drosophila* *polo*, budding yeast *CDC5*, and murine *plk* genes suggested that it should be possible to isolate a homolog from the fission yeast *S. pombe* using PCR. We therefore designed suitable primers corresponding to a sequence in the kinase subdomain VII (Hanks et al. 1988) and a conserved region present in the carboxy-terminal domain of all of the other known polo homologs (the arrows in Fig. 1) and used these to amplify segments of *S. pombe* genomic DNA. The amplified sequences were cloned and enabled us to isolate and sequence the corresponding genomic DNA. The sequence reveals a gene, which we have called *plol*⁺, that can encode a peptide of the polo-like kinase family.

We have mapped the *plol*⁺ gene on the *S. pombe* chromosomes by hybridization to a set of aligned cosmid and

P1 phage DNAs, utilized for the physical mapping of the *S. pombe* genome (Hoheisel et al. 1993). *plol*⁺ is located on the left arm of the first chromosome in a region for which no known cloned genes or mutations related to cell cycle progression have been described (see Materials and methods).

The sequence of this genomic DNA contained ambiguities in relation to the extent of the *plol*⁺ open reading frame (ORF): There are two potential initiation sites preceding the kinase domain in addition to another upstream ORF. To determine the correct initiation codon, we chose to connect the *plol*⁺ genomic sequence to conditional promoter of *nmt1*⁺ gene (Maudrell 1993) such that it could be expressed from either of these potential initiation codons. We wished to compare the sizes of the proteins expressed in this way with the mobility of the wild-type *plol*⁺ protein, thus necessitating an antibody that would recognize *plol*⁺ by immunoblotting. This was obtained by expressing the carboxy-terminal 360 amino acids of *plol*⁺ in *Escherichia coli* as a fusion protein using T7 RNA polymerase system (Studier and Moffat 1986). Rabbits immunized with this protein produced an antiserum that—both before and after affinity purification on the fusion protein—recognized a protein of the predicted size in wild-type *S. pombe* extracts (see Materials and methods for details). When the *nmt1* promoter was placed just upstream of the second potential initiation codon, a protein with the same mobility as the *plol*⁺ product was overexpressed only following derepression of transcription, thus identifying the correct initiation codon. When we expressed a segment of genomic DNA containing 4.5 kb of upstream sequence, including the second ORF on a multicopy plasmid, this resulted in the overproduction of protein of the same size as from the shorter genomic DNA. Thus, this excludes the possibility that the upstream ORF is an upstream exon.

Overall, the identity between *plol*⁺ and the respective *CDC5* and polo kinases of *Saccharomyces cerevisiae* and *Drosophila melanogaster* is ~40%, with the greatest homology (50%–60% identity) being in the catalytic do-

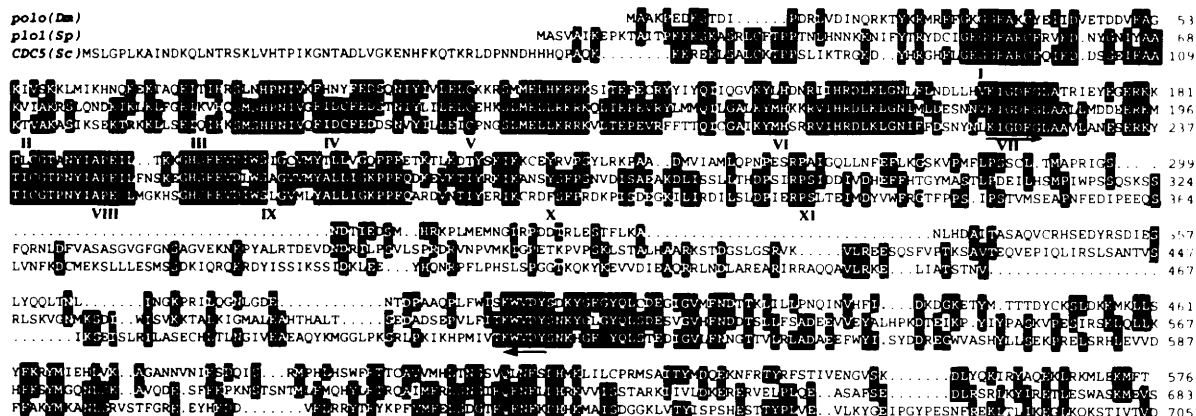


Figure 1. Comparison of amino acid sequences among polo kinase homologs. Comparison of the amino acid sequences of *D. melanogaster* *polo*, *S. pombe* *plol*⁺, and *S. cerevisiae* *CDC5*. Identical residues are marked. Arrows show the conserved amino acid sequences used to design primers for PCR. Roman numerals indicate kinase consensus subdomains (Hanks et al. 1988).

main (Fig. 1). As many kinases are themselves known to be controlled by protein phosphorylation, we have searched for potential phosphorylation sites. *Drosophila* polo is phosphorylated and is inactivated by phosphatase treatment (A. Tavares, D.M. Glover, and C.E. Sunkel, in prep.). The predicted *plol*⁺ sequence has one potential phosphorylation site for p34^{cdc2} in the amino-terminal region preceding the kinase domain, but the significance of this is not clear as the site is not conserved in the enzymes from other organisms. We note also that *plol*⁺ has a threonine residue located between domains VII and VIII (Hanks et al. 1988) conserved among all known polo homologous kinases. This corresponds to the region in which there are crucial phosphorylation sites required for the activation of kinases, such as *cdc2*, MAP kinase, and MAP kinase kinase (Ducommun et al. 1991; Gould et al. 1991; Payne et al. 1991; Alessi et al. 1994; Zheng and Guan 1994).

Loss of function of *plol*⁺

To examine the *in vivo* function of *plol*⁺ in *S. pombe*, we carried out gene disruption by one-step gene replacement (Rothstein 1983). In the diploid, one copy of *plol*⁺ was replaced by *ura4*⁺, a structure that was confirmed by genomic Southern hybridization (Materials and methods). The promoter region from position -235 and the whole ORF except the 13 carboxy-terminal amino acids were deleted. Tetrad analysis demonstrated that *plol*⁺ is essential for viability. *Ura*⁺ spores that lack the *plol*⁺ gene germinate but usually do not divide or may, in some cases, divide only once. The lethality of the *plol* disruptant can be rescued by introducing the *plol*⁺ genomic DNA or the *plol*⁺ gene driven from the *nmt1* promoter, confirming that lethality is the result of the deletion of *plol*⁺.

We have examined the cellular phenotypes resulting from the loss of *plol*⁺ by two methods: spore germination (Hagan et al. 1988) and a "switch-off" experiment in which *plol*⁺ expression from the *nmt1* promoter is repressed by addition of thiamine. In the spore germination method, diploids in which one copy of *plol*⁺ was replaced by the *ura4*⁺ gene were sporulated, and after digestion of ascus walls, which also kills the remaining cells, the spores were cultured in media lacking uracil to allow only the disruptants to grow. DNA staining with DAPI reveals three types of abnormal cells: those with one cluster of overcondensed chromosomes (Fig. 2a,b); unseptated cells with two interphase nuclei (Fig. 2c,d); and unseptated cells with two clusters of overcondensed chromosomes (Fig. 2e,f). Most cells are bulbous at one end, indicating that abnormalities have occurred before the first cell division as the cell extends from the ellipsoid spore. We have followed the time of appearance of these phenotypes in germinating spores following inoculation into culture medium (Fig. 2g). This showed that unseptated cells with two clusters of condensed chromosomes began to appear a few hours later than the other phenotypes, suggesting that these cells arrest in the second mitosis rather than in the anaphase of the first mi-

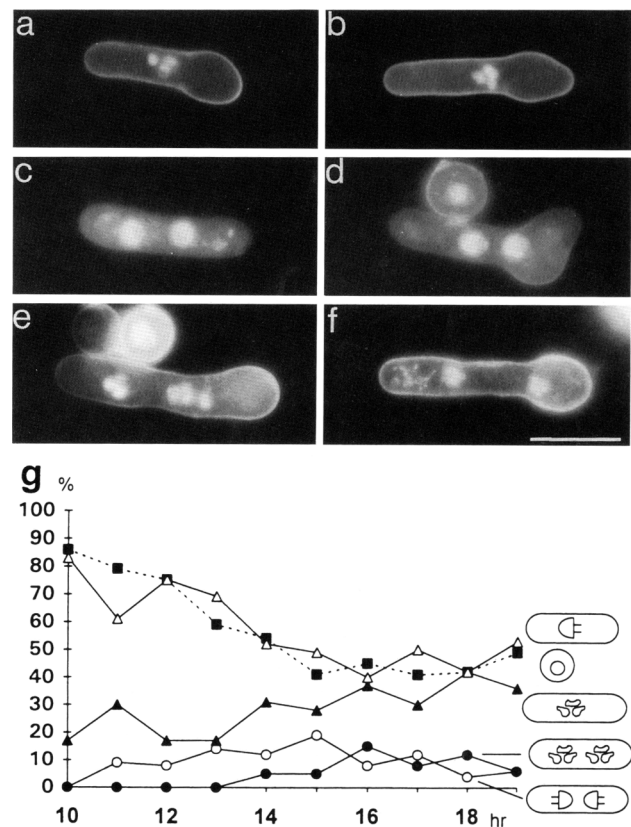


Figure 2. Defective phenotypes in *plol* disruptants. (a–f) Defective phenotypes of germinating *plol*-deleted spores. Spores from a diploid in which one copy of *plol*⁺ was replaced by *ura4*⁺ were germinated in the medium lacking uracil at 30°C to allow only the disruptant to grow. Typical defective phenotypes seen by DAPI staining are shown. (a,b) Cells with one cluster of overcondensed chromosomes; (c,d) unseptated cells with two interphase nuclei; (e,f) unseptated cells with two clusters of overcondensed chromosomes. Bar, 10 μ m. (g) Time course of the cellular phenotype following the germination of *plol*-deleted spores. The frequencies (■) of spores that have not started elongating in the total population were determined by DAPI staining. Other classes of the cells are shown as the relative frequencies among the cells that have started elongating; (△) Cells with a single interphase nucleus; (■) cells with one cluster of overcondensed chromosomes; (○) unseptated cells with two interphase nuclei; (●) unseptated cells with two clusters of overcondensed chromosomes.

tosis. The results suggest that most germinated disruptant spores become blocked in their first mitosis. Disruptants that proceed through the first mitosis fail to form a septum and are then blocked in the second mitosis.

To follow the consequences of switching-off of *plol*⁺ expression in growing cells, we utilized haploid cells with a disrupted chromosomal *plol*⁺ gene, which were kept alive by an episomal *plol*⁺ gene under the control of the modified weaker *nmt1*⁺ promoter in the absence of thiamine (pREP81; Basi et al. 1993). Under these conditions we observe cell cycle progression with no obvi-

ous cellular abnormalities. We then studied the time course of the cellular phenotypes following the addition of thiamine to repress *plol*⁺ transcription. As in the spore germination experiment, we could observe both septation and mitotic defects, but in the switch-off experiment, the septation defect is predominant. Nearly half of the cells lack septa and have multiple nuclei (up to 8) 23 hr after addition of thiamine (Fig. 3), suggesting that after failure of septum formation, one or two nuclear divisions have taken place before the cells become blocked in mitosis. A relatively small proportion of the unseptated cells show overcondensed chromosomes.

These two types of experiment indicate that *plol*⁺ is required for mitotic progression and septum formation. In contrast to the spore germination experiment, the gene product is expected to be gradually diluted out in cells in the switch-off experiment. Thus, the difference observed in the proportions of the different cellular phenotypes between the two methods probably reflects different thresholds of gene activity required for the two functions. It suggests that a lower level of *plol*⁺ activity is required for mitosis than for septum formation.

plol⁺ is required for the formation of a bipolar spindle

To analyze the mitotic defects resulting from the loss of *plol*⁺ function, we examined microtubules and spindle pole bodies (SPBs) stained with the anti- α -tubulin monoclonal antibody (TAT1; Woods et al. 1989) and an anti-sad1⁺ polyclonal antibody (Hagan and Yanagida 1995), respectively. We found that both following the germination of disruptant spores and after the switch-off of

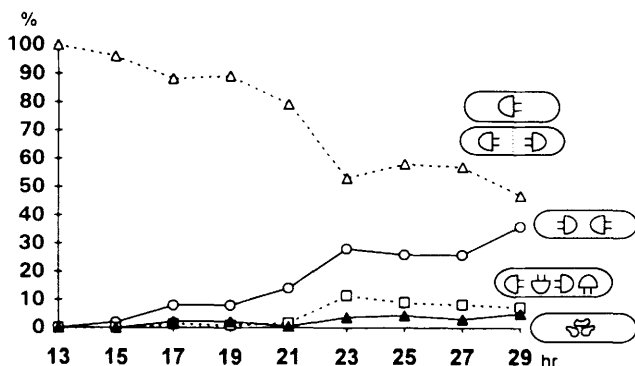


Figure 3. Time course of the cellular phenotype upon switching-off *plol*⁺ expression. Disruptant cells are kept alive by the expression of *plol*⁺ from the conditional promoter of *nmt1*⁺. Defective phenotypes were examined by DAPI staining after *plol*⁺ switch-off following the addition of thiamine to the culture. The relative frequencies of each phenotype are shown; (Δ) Apparently normal cells either in interphase and mitosis; (○) unseptated cells with two interphase nuclei (usually back to back orientation); (□) unseptated cells with more than two interphase nuclei (cells with 4 nuclei are major); (▲) cells with overcondensed chromosomes. In this method, the septation defect is more emphasized. Up to 8 nuclei per cells can be observed. One or more clusters of overcondensed chromosomes are seen in a small proportion of cells.

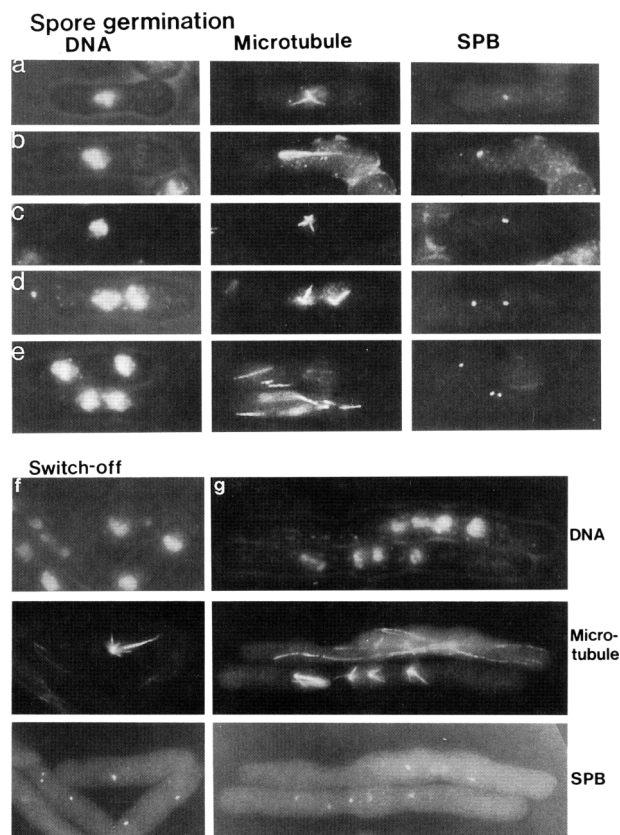


Figure 4. Microtubule and SPB staining of *plol*⁺ disruptants. (a-e) Spore germination; (f,g) Switch-off experiments. Overcondensed chromosomes are associated with monopolar spindles (a-d and the lower cell in g). A very minor fraction of nuclei show abnormal chromosome segregation despite association with bipolar spindles (e.g., the left-most set of nuclei in the bottom cell in g). Some nuclei associated with a bipolar spindle show no segregation of chromosomes. Others show spindle elongation without chromosome segregation. Unseptated cells with multiple interphase nuclei have interphase microtubules (e and the top cell in g). Note that a SPB associated with the right-most nucleus of the top cell in e and some SPBs of the top cell in g are out of focus. The SPBs of at least some monopolar spindles appear to have duplicated (b,c). Examples of normal microtubules/SPB configurations can be seen in Fig. 6C. Bar, 10 μ m.

plol⁺ expression in exponentially growing cells, overcondensed chromosomes were associated with defective spindles nucleated from single poles (the sad1-staining dots in Fig. 4). Typically, one to several strong wedge-shaped microtubule structures emerged from the SPBs to pass through the cluster of the overcondensed chromosomes (Fig. 4a,b). The defective spindle structure resembles that seen with mutation of the *cut7*⁺ gene, which encodes a member of the kinesin family required for SPB separation. A discrete SPB structure is stained by the anti-sad1⁺ antibody, although the staining of some cells suggests that SPB duplication might have taken place (e.g., Fig. 4c). Unseptated cells may have multiple nuclei

either in interphase or mitosis (Fig. 4d–g). In the former, the cells have interphase arrays of microtubules; in the latter, most condensed chromosomes are associated with a monopolar spindle.

plol⁺ is required for both actin ring formation and septum deposition

Previous genetic and cytologic analyses have shown that septum formation requires two at least partially separable processes, actin ring formation and deposition of septal material (Nurse et al. 1976, Marks et al. 1986, 1987). In the “late” septation mutants (*cdc3,cdc4,cdc8,cdc12*), actin ring formation is defective but septal material is deposited in disorganized manner. In the “early” septation mutants (*cdc7,cdc11,cdc14,cdc15*), septal material is not deposited, but at least in the case of *cdc11* and *cdc14*, the actin ring is formed during mitosis. To examine the nature of the septation defect in the *plol* disruptant, we first examined F-actin localization. In wild-type *S. pombe* cells, F-actin is localized predominantly at the old (growing) end of the newly divided cell. After completion of one-third of the cell cycle, F-actin is found at both ends of the cell, both of which grow throughout the rest of interphase. With mitotic commitment, F-actin disappears from both ends of the cell and an actin ring starts to be formed at the equator (Marks and Hyams 1985), persisting until cell separation. Staining of F-actin with rhodamine-conjugated phalloidin reveals the actin ring structure at the equator of all wild-type anaphase cells (Fig. 5a). However, when we examined F-actin distribution following the switch-off of *plol*⁺ expression from the *nmt1* promoter (as described above), we found that (20 hr after the addition of thiamine) only 18% of the anaphase cells has developed an F-actin ring, whereas 68% of the cells showed dispersed dots of actin on the cell surface (Fig. 5b,c). In the control culture (the same culture maintained in the absence of thiamine), all anaphase cells showed an actin ring. This demonstrates that loss of *plol*⁺ leads to defects in actin ring formation. Unseptated cells with two interphase nuclei (oriented back to back) usually had F-actin dots at both ends (data not shown). Thus loss of *plol*⁺ function does not appear to affect the localization of F-actin during interphase. We also examined septal material deposition by staining cells with Calcofluor and DAPI following the switch-off of *plol*⁺ expression. We found that even in unseptated cells with multiple nuclei there was no significant deposition of septal materials that could be stained brightly with Calcofluor (Fig. 5d). Thus, in summary, loss of *plol*⁺ disrupts both actin ring formation and septal material deposition, both of which are known to be required for septum formation and cell cleavage.

plol⁺ overexpression results in mitotic defects and septum formation without nuclear division

To test the consequences of overexpressing *plol*⁺, we connected the gene to the *nmt1* promoter or its derivatives and introduced it into wild-type cells on a multi-

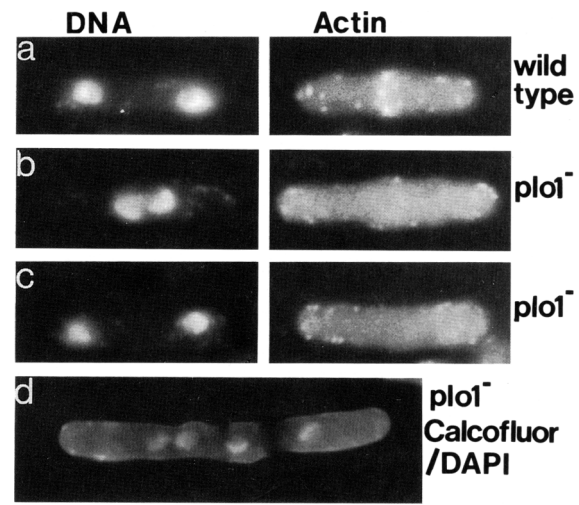
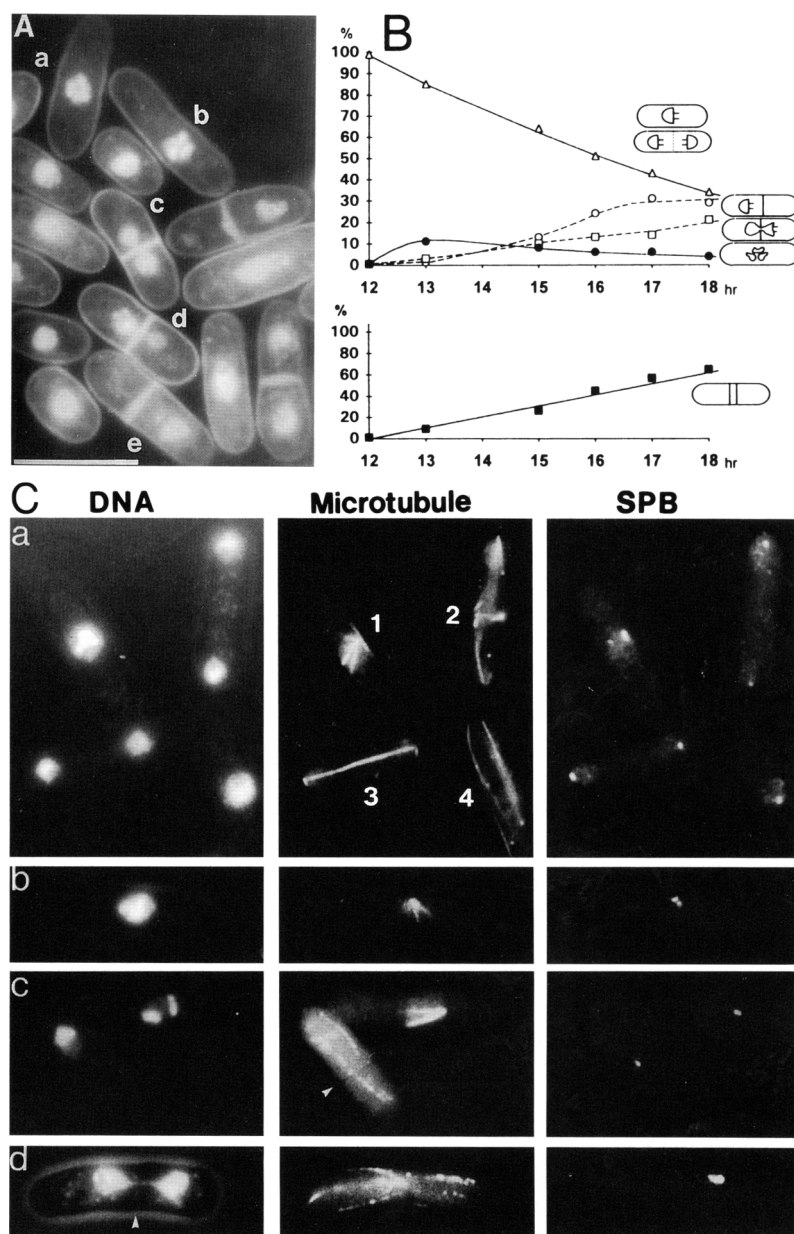


Figure 5. F-actin and septal material staining of *plol* disruptants. (a–c) DNA and F-actin were stained using DAPI and rhodamine-conjugated phalloidin, respectively. Wild-type cells with dividing nuclei always have an F-actin ring (a). In the switch-off experiments (b,c), cells were stained 20 hr after addition of thiamine. In contrast, 68% of *plol*-depleted cells with dividing nuclei have dispersed F-actin dots instead of the actin ring (b,c). (d) Septal material and DNA of the *plol*-depleted cells from the same switch-off experiment were stained using Calcofluor and DAPI, respectively. Most unseptated cells with two or more nuclei do not show any deposited septum material that would be stained very brightly by Calcofluor. Bar, 10 μ m.

copy vector. Overexpression of the *plol*⁺ transcript from the wild-type *nmt1* promoter (in the pREP1 plasmid) inhibits colony formation of wild-type cells on plates. When expressed from a mutated 10-fold weaker promoter (in the plasmid pREP41), there was no significant effect on colony size.

Before examining the nature of this phenotype, we first monitored the production of *plol*⁺ product in liquid culture by carrying out Western blotting of cell extracts made at timed intervals following the stimulation of transcription by the withdrawal of thiamine. At 32°C, the *plol*⁺ products start to accumulate from ~10 hr and plateau at maximum levels at 14 hr from the time of thiamine withdrawal. Maximum *plol*⁺ protein levels are ~10 times that in a strain expressing *plol*⁺ gene from its own promoter on a multicopy vector. When we stained cells overexpressing *plol*⁺ with DAPI we found two principal defective phenotypes: cells that have overcondensed chromosomes (Fig. 6A, cells a and b) and cells having a single septum or multiple septa and one interphase nucleus. In the latter case, the nucleus is either pinched by the septum (Fig. 6A, cells c and d) or dislocated to one side of the septum (Fig. 6A, cell e). However, unlike the *cut* mutants (Hirano et al. 1986), cell separation rarely follows, and, instead, cells with multiple septa accumulate (Fig. 6B, bottom graph). When expressed from the weaker promoter (in REP41), 11% of cells show similar defects. No abnormalities were ob-

Figure 6. Overexpression of *plol*⁺ in wild-type cells. (A) DNA staining. Cells with pHN204 (*nmt1*⁺-*plol*⁺) were stained with DAPI after withdrawal of thiamine to induce *plol*⁺ transcripts from the *nmt1*⁺ promoter. Two types of abnormalities are seen: cells with overcondensed chromosomes (a and b) and septated cells with single interphase nuclei. The latter can be classified into cells in which the nucleus is pinched with the septum (c and d) and cells in which the nucleus is dislocated to one side of the septum (e). Bar, 10 μ m. (B) Time course. The relative frequencies of each category of cells were determined. (Δ) Apparently normal cells in either interphase or mitosis; (\bullet) cells with overcondensed chromosomes; (\square) cells with an interphase nucleus pinched by a single septum or multiple septa; (\circ) cells with an interphase nuclei to one side of the septum (septa). The bottom graph (\blacksquare) shows the frequency of septated cells that have multiple septa in the same culture determined by Calcofluor staining. Only clear mutiseptated cells were scored. Cells are cultured at 32°C. (C) DNA, microtubules, and SPB staining. DNA, microtubules, and SPBs were immunostained using DAPI, the anti α -tubulin antibody TAT1, and anti-sad1⁺ antibody. Normal interphase (cell 4), anaphase (cell 3), and telophase cells (cell 2) can be seen in the population. Overcondensed chromosomes are associated with monopolar spindles (cell 1 in a,b and the top cell in c). Septated cells with a single interphase nucleus have interphase microtubules (the left cell in c and d). Arrowheads show the position of septa. Bar, 10 μ m.



served in the presence of thiamine, which represses the *nmt1* promoter. We have also overproduced the carboxy-terminal noncatalytic half of *plol*⁺ using the *nmt1* promoter and find that this leads to no abnormalities.

When we examined the microtubules and SPBs in such cells, we found overcondensed chromosomes associated with defective spindles nucleated from a single dot of SPB staining [Fig. 6C(a–c)]. As with the disruptant, one to several wedge-shaped microtubule structures emerge from the SPB and penetrate through the cluster of overcondensed chromosomes. Furthermore, in some cells the staining pattern suggested that the SPB appeared to be duplicated but not separated [e.g., Fig. 6C(c)]. On the other hand, those cells with a single interphase nucleus

and one or more septa have an interphase arrangement of microtubules [Fig. 6C(c,d)], in which the intensity of microtubule staining is often diminished over that seen in comparable wild-type populations.

plol⁺ overexpression can induce septum formation in *G*₂ cells

As loss of *plol*⁺ affects both “early” and “late” pathways of septum formation, this suggests that *plol*⁺ kinase has an upstream function in the septum formation pathway. To test whether *plol*⁺ can overcome the prior requirement of the initiation of mitosis for septum formation, we examined the effects of its overproduction in

the *cdc25-22^{ts}* mutant that arrests in late G₂ at restrictive temperature. We introduced the pREP1 plasmid carrying *plp1*⁺ under the *nmt1* promoter into this strain and examined the effects of overexpression of *plp1*⁺, shifting to the restrictive temperature for *cdc25-22* following 14 hr in medium lacking thiamine. Overproduction of *plp1*⁺ under these conditions results in septum formation (Fig. 7a,c) in the absence of (<0.5%) nuclear division, chromosome condensation, and spindle formation. We were concerned that the induction of septation

could reflect leakiness from the *cdc25-22* arrest. Half of the culture was therefore subjected to a parallel temperature-shift procedure without withdrawing thiamine from the medium. The frequency of the cells with septa increased from <20% to >80% between 2 and 5 hr following the temperature shift, whereas in a period between 1 and 8 hr following the temperature shift in the control culture, the cell number increases by <25%. Thus, leakiness of the *cdc25-22* arrest under these conditions is insufficient to explain septum formation.

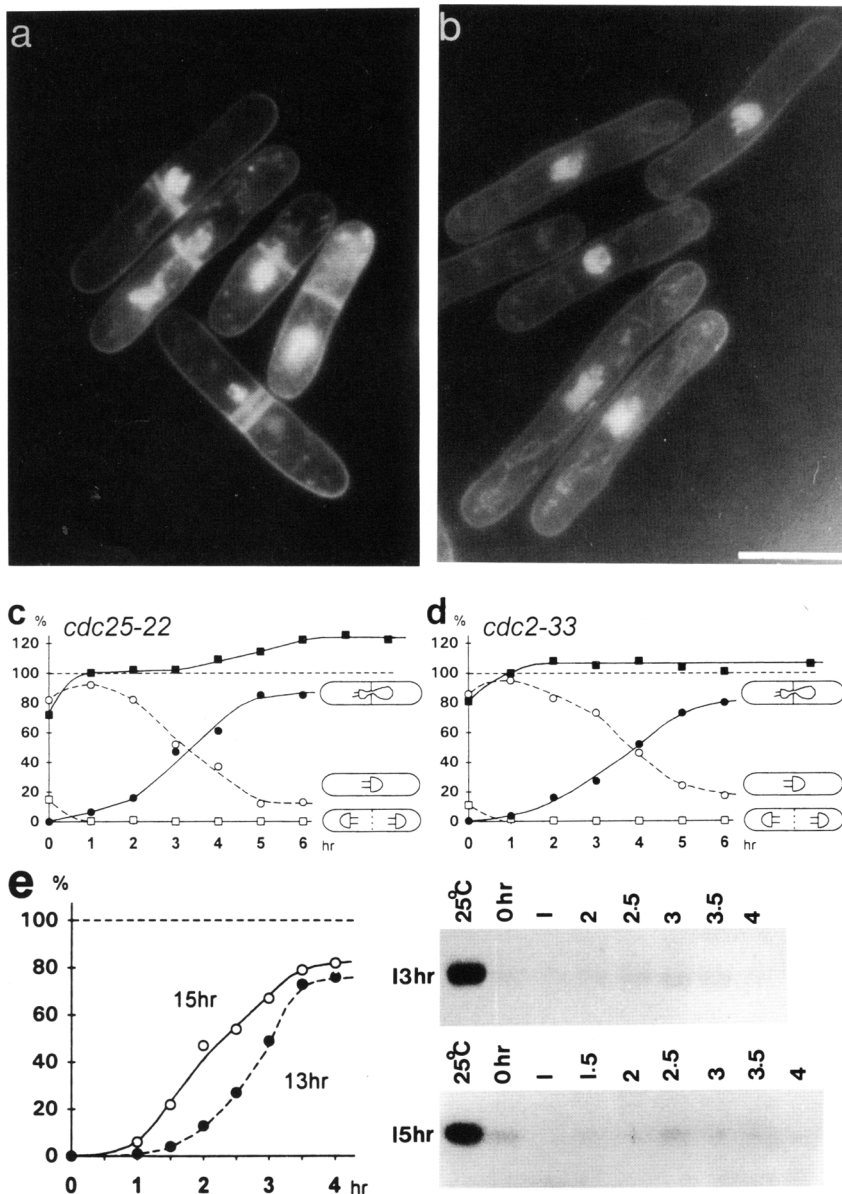


Figure 7. Overexpression of *plp1*⁺ in G₂-arrested cells. (a,b) DNA staining of *cdc25-22* arrested cells that either overexpress *plp1*⁺ (a) or do not (b). Cells from the culture with (a, induced) or without thiamine (b, noninduced) were stained with DAPI 3.5 hr after shift-up following the procedures given below. Bar, 10 μm. (c,d) Time course of septum induction during the overexpression of *plp1*⁺ in *cdc25-22*-arrested (c) or *cdc2-33*-arrested (d) cells. Aliquots of the culture [strains carrying pHN204 (*nmt1-plp1*⁺)] were taken for DAPI staining after shift-up (0 time) to restrictive temperature, 36°C, following 14-hr culture in the medium lacking thiamine at permissive temperature, 25°C. The frequencies of each class were shown. In both cases, cells with two nuclei (○) had disappeared within 1 hr after shift-up. Septated cells with a single nucleus (●) accumulated rapidly, whereas unseptated cells with a single nucleus (○) decreased. For the control, half of the culture was subjected to the same shift-up procedure in parallel without thiamine withdrawal. Leakiness from the arrest was monitored by counting the increase in cell number of the control (■). Chromosome condensation or spindle formation (determined by immunostaining) was rarely (<0.5%) observed later than 1 hr. However, it was sometimes difficult to determine chromosome condensation after prolonged arrest (>5 hr) because of the distortion of nuclear morphology in both induced or noninduced cultures. (e) Time course of septum induction during the overexpression of *plp1*⁺ in *cdc25-22* cells following enrichment of G₂ cells. Asynchronous *cdc25-22* cells carrying pHN204 were size-fractionated by two successive centrifugations through lactose gradients following cultures without thiamine for 13 hr (●) or 15 hr (○). No or few (<1%) abnormal cells were detected in the starting asynchronous cultures. Small cells

corresponding to early G₂ cells were taken and inoculated (0 time) into the prewarmed medium lacking thiamine at 36°C. At 0 time, no septa were observed at all. Aliquots of the cultures were taken at regular time intervals for DAPI staining, microtubule/SPB staining, and p34^{cdc2} kinase assay. The frequencies of septated cells with a single nucleus are shown in the graph. No spindle formation was observed. p34^{cdc2} kinase activities of each sample were assayed using histone H1, together with asynchronous culture at permissive temperature just before fractionation (25°C), as described in Moreno et al. (1989), and the radiolabeling of histone H1 was detected by autoradiography as shown. No activation of p34^{cdc2} activity was observed despite the rapid induction of septum formation.

The leakiness from a *cdc25-22* arrest is considered to be caused by prolonged arrest in the G₂/M boundary (Hagan and Hyams 1988). To minimize the length of time during which cells are arrested in the G₂/M boundary and yet allow sufficient time for the temperature-sensitive *cdc25-22* product to be inactivated, we examined the effects of *plol*⁺ overexpression in the *cdc25-22* mutant in a G₂-enriched population of cells. We subjected asynchronous cultures to size fractionation by centrifugation through lactose gradients following culture for either 13 or 15 hr in medium lacking thiamine. The small early G₂ cells were collected from the gradients and shifted immediately to 36°C. Examination of aliquots of cells taken at regular intervals indicated that neither spindle formation, chromosome condensation, nor p34^{cdc2} activation occurred, and yet septum formation was induced rapidly (Fig. 7e). The culture in which *plol*⁺ expression had been derepressed for 15 hr at 25°C underwent septation ~1 hr ahead of the culture derepressed for 13 hr.

The above experiment also suggests that overproduction of *plol*⁺ itself is not activating p34^{cdc2} kinase and so forcing cells into a mitotic state. To confirm this we also examined the effects of overexpressing *plol*⁺ in *cdc2-33*-arrested cells following temperature shift of an asynchronous culture. The result was very similar to that seen with *cdc25-22*. The percentage of cells that have septa and a single nuclei increased from 5% to 75% between 1 and 5 hr, whereas the cell number increased in the control culture by only 10% (Fig. 7d). This result strongly suggests that p34^{cdc2} activation is not required for induction of septum by overexpression of *plol*⁺.

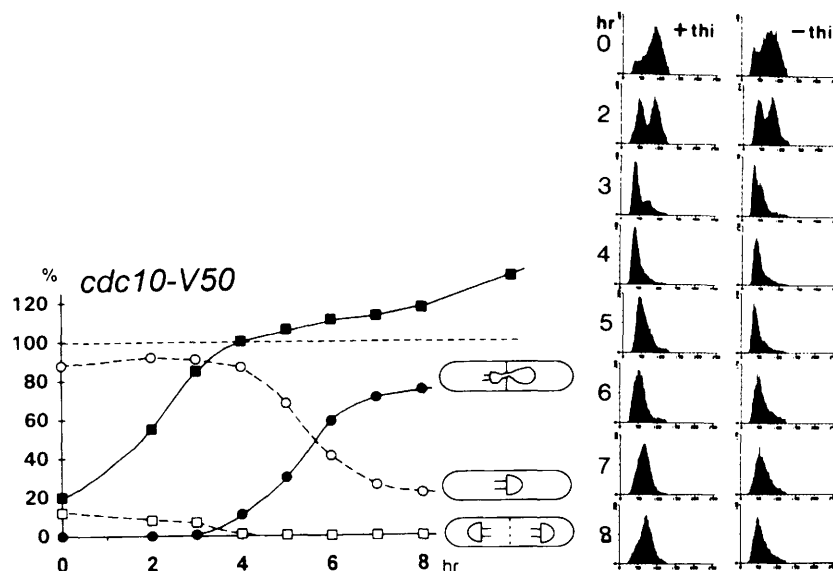
plol⁺ overexpression can induce septum formation in G₁ cells and cycling cells that lack M phase

We then asked whether overproduction of *plol*⁺ would also induce septation in G₁ cells. We therefore intro-

duced the plasmid that can overexpress *plol*⁺ into the *cdc10-V50* mutant (Marks et al. 1992) that arrests prior to Start in G₁ at the restrictive temperature of 36°C (Fig. 8). As the execution point of *cdc10* mutants is at ~0.1 of a cell cycle (Nurse et al. 1976), to arrest the mutant completely, we shifted an asynchronous cell population to 36°C after 12 hr in medium lacking thiamine, somewhat earlier than in the previous experiments. We monitored the effectiveness of the block using flow cytometry to measure DNA content of the cells. Nearly all cells become arrested in G₁ 4 hr following the temperature shift in cultures grown in the presence or absence of thiamine. In the culture overproducing *plol*⁺, the frequency of septated cells increases from 10% to 70% between 4 and 7 hr following the temperature shift. At 7 hr, 70% of these cells had a single nucleus and had formed a septum and the greater majority of cells had a G₁ DNA content. This excludes the possibility that the leakage from the block is required for the induction of septation, and, so, indicates that overexpression of *plol*⁺ can induce septum formation not only in G₂-arrested cells but also in G₁-arrested cells.

Recently Hayles et al. (1994) demonstrated that germinating spores lacking the *cdc13* gene do not undergo mitosis and, instead, undertake repetitive S phases with intervening G phases. We therefore examined the consequences of overexpressing *plol*⁺ in the *cdc13* disruptant in which actively cycling cells are unable to enter mitosis. We found that this leads to the formation of long septated cells containing large nuclei, indicating that the cells have undergone repetitive S phases, typical of the *cdc13* disruptant. We observed that septa are formed in 49% of the long (>45 μm) cells 24 hr after inoculating spores into medium lacking thiamine, uracil, and leucine that allows only the disruptant to grow. Only 7% of the long cells show septum formation under uninduced condition (in medium containing thiamine). This experiment confirms our conclusion that *plol*⁺ overproduc-

Figure 8. Time course of the cellular phenotype and DNA content during *plol*⁺ overexpression in *cdc10-V50* cells. Procedures are identical to those used for the experiments shown in Fig. 7, a–d, except that temperature shift-up was given 12 hr after thiamine withdrawal. This allows the *cdc10-V50* cells carrying pHN204 sufficient time to arrest prior to Start in G₁ before the overexpression effects appear. The distribution of DNA content of individual cells determined by flow cytometry is shown in the histograms. In both induced or noninduced cultures, the large majority of cells arrest in G₁ though most cells in the induced culture develop septa.



tion can overcome the prior requirement for p34^{cdc2}/cyclin B activation for septum formation.

Discussion

Dual roles of *plol*⁺

We have identified an *S. pombe* gene, *plol*⁺, that encodes a kinase homologous to *Drosophila polo* and *S. cerevisiae CDC5*. The phenotypic analysis of loss-of-function and overexpression strains indicates that the *plol*⁺ gene is involved in both establishment of bipolar spindle and septum formation (Fig. 9). Though we have no evidence that these two roles are independent, they appear to be separable. Differences in the proportions of cells with mitotic or septation phenotypes following either the germination of *plol*⁺ disruptant spores or the switch-off of an inducible version of the gene in exponentially growing cells suggest differing thresholds for the *plol*⁺ kinase for its two functions. Moreover, overexpression of *plol*⁺ in interphase-arrested cells induces septum formation without other mitotic events. Are these two roles of the *plol*⁺ kinase truly independent? Certainly there is precedent for protein kinases functioning at different cell cycle stages. This is seen with the *S. cerevisiae* CDC28 kinase that functions at Start, during S phase, and at the G₂-M transition in association with different cyclin subunits. However, as formation of both the bipolar spindle and the actin ring leading to septum formation occurs at roughly the same time in the *S. pombe* mitotic cycle, it is not necessary to postulate activity of *plol*⁺ kinase at two distinct points in mitotic progression. It is not difficult to imagine that *plol*⁺ kinase has many substrates and so can regulate several mitotic processes.

Roles of *plol*⁺ in septum formation

Nurse et al. (1976) originally isolated and characterized a number of mutants defective in septum formation. One

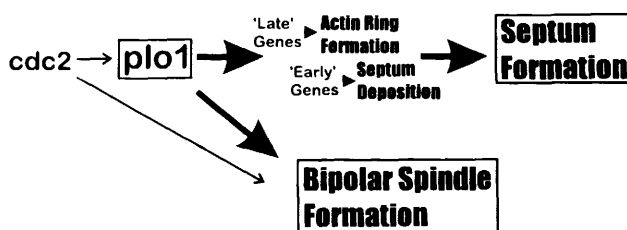


Figure 9. Proposed roles of *plol*⁺ in *S. pombe*. *plol*⁺ kinase has dual functions: establishment of the bipolar spindle and induction of septum formation. *plol*⁺ functions upstream of the whole septum formation pathway, including actin ring formation (regulated by late septation genes) and septal material deposition (regulated by early septation genes). As overexpression of *plol*⁺ can overcome the requirement of *cdc2*⁺ activation for septum formation, *plol*⁺ probably functions between *cdc2*⁺ and septum formation. *plol*⁺ function is sufficient for septum formation, but *cdc2*-dependent factors are also required for bipolar spindle formation.

category (*cdc7*, *cdc11*, *cdc14*, and *cdc15*), the early septation mutants, neither forms a septum nor accumulates septal material. The other category of mutants (*cdc3*, *cdc4*, *cdc8*, and *cdc12*), the late septation mutants, fails to form a septum but does accumulate disorganized septal material. Examination of actin distribution in wild-type cells reveals that prior to septum formation, the actin ring forms at an early stage of mitosis at the equator of the cell (Marks and Hyams 1985). Two of the early septation mutants, *cdc11* and *cdc14*, have been examined and found to show actin ring formation in mitosis (Marks et al. 1986; Fankhauser and Simanis 1994b). On the other hand, in the late septation mutants, actin ring formation is defective and septal material is deposited at a position that correlates with the disorganized actin distribution (Marks et al. 1987). Consistently, recent molecular analysis shows that at least two of the late septation genes encode components of the actin contractile ring, namely *cdc3*⁺ and *cdc8*⁺, which encode profilin and tropomyosin, respectively (Balasubramanian et al. 1992, 1994). These results suggest that septum formation requires two pathways, actin ring formation (regulated by late septation genes) and the deposition of septal material (regulated by early septation genes) and that the two pathways are, at least partially, separable. Our cytological analysis of *plol* disruptants indicates that *plol*⁺ is required for both actin ring formation and septal material deposition, suggesting that *plol*⁺ kinase is likely to function upstream of both early and late septation genes. Of the early septation genes, *cdc7*⁺ encodes a kinase and has been claimed as a key regulator of septum formation on the basis that loss of its function causes defects in septum formation, its overproduction results in multiseptum formation, and its overproduction can suppress the septum formation defect of *cdc11* (Fankhauser and Simanis 1994a). In contrast to the phenotype seen in the overexpression of *plol*⁺ however, overexpression of *cdc7*⁺ will not drive G₂-arrested cells into septation, and thus, it cannot overcome the normal dependency of septation upon the onset of mitosis (Fankhauser and Simanis 1994a).

The ability of *plol*⁺ to induce septum formation in interphase cells following its overexpression does not require the activation of p34^{cdc2}/cyclin B. As with cytokinesis in other eukaryotes, actin ring formation and septation in *S. pombe* are dependent on the prior initiation of mitosis. No mutants, drugs, or other factors have been reported previously to overcome this dependency in any eukaryotes. The finding that *plol*⁺ function is both essential and sufficient for septum formation leads us to propose that *plol*⁺ is the key rate-limiting inducer of the whole pathway for septum formation including actin ring formation. In short, *plol*⁺ kinase behaves as a "septum-promoting factor" in *S. pombe*.

Two models can explain how *cdc2* kinase can regulate a wide variety of mitotic events. On the one hand, *cdc2* kinase might directly regulate each mitotic event. Alternatively, but not exclusively, it might directly or indirectly activate a set of mediators, each of which induces each mitotic event. *plol* kinase appears to act as such a

mediator because it is essential for the septum formation pathway, and its overexpression can overcome the prior requirement for *cdc2*⁺ activation in this process. Although a number of genes essential for mitotic progression have been identified, the NIMA kinase of *Aspergillus* is the only candidate for a mediator other than *plp1*⁺. Overproduction of stable NIMA kinase can induce chromosome condensation in interphase cells (O'Connell et al. 1994). However, NIMA appears to be required for all aspects of mitosis. None of the putative *nimA* homologs reported from other organisms have any homology outside of the kinase catalytic domain, which casts some doubt on their assignment as true *nimA* homologs.

In addition to its function as a septum-promoting factor, *plp1*⁺ could also be involved in inducing other late events of cell division. It will be of future interest to examine whether *plp1*⁺ is involved in inducing events such as the reorganization of microtubules into the post-anaphase array, nuclear repositioning, chromosome decondensation, actin relocalization, and the reinitiation of the next cell cycle.

Roles of plp1⁺ in mitosis

Mitosis consists of a series of temporally and spatially coordinated events. As in higher eukaryotes, the major mitotic events, such as chromosome condensation, spindle formation, metaphase alignment, anaphase A, and anaphase B, are well defined in *S. pombe* (Toda et al. 1981; Hiraoka et al. 1984; Hagan et al. 1990; Funabiki et al. 1993). Moreover, mitotic regulation in *S. pombe* is genetically the most well studied of all organisms. Many *S. pombe* mutants affecting specific mitotic events and their regulation have been isolated (Yanagida 1989), and their analysis is leading to the identification of molecular mechanisms for mitotic progression. However, although phosphorylation is a common way to regulate protein activity and several kinases are known that regulate the onset of mitosis (Nurse 1990), *plp1*⁺ is the first kinase to be identified that is essential for mitotic progression in *S. pombe*.

Both loss of function and overexpression of *plp1*⁺ gene result in monopolar spindles, suggesting that the proper level or timing of activation of the kinase is required to establish a bipolar spindle. There are at least two possible means of generating monopolar spindles, either through defects in SPB duplication or in the separation of duplicated SPBs. In *plp1* disruptants, the *sad1* staining suggests, in some cases, that the SPB might be at least partially duplicated at the resolution level offered by immunofluorescence microscopy. It is equally possible that SPB duplication has not been completed. When and how the SPB is duplicated in the *S. pombe* cell cycle is not yet established. The *cut7*⁺ gene, which encodes a spindle-associated kinesin-like protein, is required for SPB separation (Hagan and Yanagida 1990, 1992; I.M. Hagan, K. Tanaka, and M. Yanagida, unpubl.). *cut7* mutants show similar mitotic phenotype to that of *plp1* disruptant or overexpressing strains as revealed by fluorescence microscopy. In this mutant, microtubule bundles nucleated

from the SPBs fail to interdigitize with one another (Hagan and Yanagida 1990). Recently, we have found a genetic interaction between *plp1*⁺ and *cut7*⁺ (I.M. Hagan, unpubl.) suggesting that *cut7*⁺ may have a close functional relationship with *plp1*⁺. It will be of future interest to test whether *cut7*⁺ is a substrate of *plp1* kinase or whether *plp1*⁺ affects the function or localization of *cut7*⁺. There appear to be parallels in *Drosophila* where the most striking effect of the original *polo* allele is on spindle structure, including the generation of monopolar spindles (Llamazares et al. 1991). Monopolar spindles in *Drosophila* can be generated by mutations in *aurora*, which also encodes a protein kinase (Glover et al. 1995); in *merry-go-round* (Gonzalez et al. 1988), which is not yet cloned; and in *KLP61F*, which encodes a kinesin-like protein closely related to *cut7*⁺ (Heck et al. 1993). However, the relationship between the products of these genes, specifically whether either of these kinases can phosphorylate and, so, modify the function of the kinesin-like protein, is not yet known. However, it is not inconceivable that the regulation of spindle function by phosphorylation of kinesin-like proteins may be conserved among eukaryotes.

In contrast to its effect on septum formation, overexpression of *plp1*⁺ is insufficient to induce spindle formation in interphase cells. *plp1*⁺ has to work in concert with *cdc2*⁺ and/or some other downstream mediators to form the bipolar spindle. Masuda et al. (1992) demonstrated that incubation of permeabilized *S. pombe* interphase cells with a *Xenopus* mitotic extract could activate the microtubule nucleation activity of the SPB resulting in the formation of a monopolar spindle. This did not happen using an interphase extract. Thus, the nucleation of spindle microtubules from the SPBs is required prior to the establishment of the bipolar spindle identifying the need for *cdc2* function. Moreover, in these experiments, purified MPF alone was insufficient to activate SPBs consistent with the requirement for mediating spindle-activating factors such as *plp1*⁺.

Conservation of plp1⁺ function

Genes with high sequence similarity to *Drosophila polo* have been identified from various eukaryotes (see introductory section). These encode kinases that not only share high similarity in the catalytic domain but also in the carboxy-terminal half of the molecule. This extension of identity beyond the catalytic domain strongly suggests that *plp1*⁺ is the *S. pombe* homolog of *polo*, although it is still possible that they are close family members, in the sense of the relationship between *cdc2*⁺ and the other *cdks*. The relative degree of identity between the polo-like kinases from different organisms suggests that they are homologs. However, in the mouse, one (Snk; Simmons et al. 1992) of the two polo-like kinases that have been identified seems to be a more distantly related family member from its sequence.

Conserved proteins may or may not show functional exchangeability across species barriers. The more important question, however, is whether the polo-like kinases

have equivalent biological functions in different organisms. There is a yet little direct information on the biological function of the vertebrate polo homologs. However, the abundance of their transcripts in proliferating tissues (Clay et al. 1993; Lake and Jelinek 1993; Golsteyn et al. 1994; Hamanaka et al. 1994; Holtrich et al. 1994), together with the cell cycle changes in the level and localization of human *plk1* (Golsteyn et al. 1994) suggest a cell cycle role.

In contrast, genetic analysis in *Drosophila*, budding yeast, and fission yeast provides considerable information about biological function. Mutants of *Drosophila polo*, *S. cerevisiae cdc5*, and *S. pombe plo1* all show mitotic defects (Hartwell et al. 1973; Sunkel and Glover 1988; this paper). However, the extent to which they affect similar processes is not clear. It is difficult at the present time to make comparisons because of the different nature of the mutant alleles in the three organisms and because of inherent differences of their nuclear division and cell cycles.

The *S. pombe* early septation gene *cdc7⁺* has weak similarity to *S. cerevisiae CDC15* and weakly complements *cdc15* mutation (Fankhauser and Simanis, 1994a). *CDC15* appears to be required for related events to *CDC5* in the budding yeast cell cycle in that their mutants show a similar terminal phenotype (Pringle and Hartwell 1981). Mutations in both genes lead to an arrest late in nuclear division, with chromosomes having separated on an elongated spindle; cytokinesis does not take place. The failure of cytokinesis is reminiscent of the phenotypes resulting from loss of function of the fission yeast genes *cdc7⁺* and *plp1⁺*. However, unlike the fission yeast mutants, there appears to be no effect on establishment of bipolar spindle, and, moreover, cells arrest with an intact spindle. It is possible that the absence of defects in bipolar spindle formation are the result of the very low threshold of requirement for bipolar spindle formation in *S. cerevisiae*. It could also reflect the unconventional timing of bipolar spindle formation in *S. cerevisiae* that begins during S phase (Byers and Goetsch 1974; Pringle and Hartwell 1981). Furthermore, unlike *S. pombe*, actin ring formation occurs in budding yeast after the spindle completes elongation (Adams and Pringle 1984; Kilmartin and Adams 1984). It will be of interest to further examine the phenotypes of these budding yeast mutants to determine if and how the actin cytoskeleton is affected. Thus, events, which take place roughly simultaneously in *S. pombe*, are temporally separated in the *S. cerevisiae* cell cycle. It is therefore possible that *S. cerevisiae* might have abandoned one function of its polo-like kinase or that another related kinase might have become specialized for this role, as with the divergence of *cdc2⁺* and *cdks* in mammalian cells. Although there appears not to be an effect on establishment of bipolar spindle in *S. cerevisiae* in the currently existing alleles of *cdc5*, we do note that the *CDC5* mutation may have an effect on microtubule behavior, because following the release of a temperature-sensitive *cdc5* strain from a block at the nonpermissive temperature, cells become insensitive to the microtubule depolymerizing

drug MBC (Wood and Hartwell 1982). However, the very fact that *CDC5* and *CDC15* affect similar events in the budding yeast and that their fission yeast genes *plp1⁺* and *cdc7⁺* are both required for septation suggest that the sequential action of these two genes might be required in other species to mediate a fundamental mitotic process.

In *Drosophila*, most work has been carried out on the analysis of the the original mutant allele, a hypomorph in which there is sufficient residual polo function that, together with a maternal contribution of polo, permits many homozygous mutant larvae to develop to adulthood. Homozygous mutant females produce embryos that fail to develop past the syncytial stages as a consequence of extensive mitotic abnormalities. Cells in the developing central nervous system of homozygous mutant larvae do show spindle abnormalities including monopolar spindles (Llamazares et al. 1991), a phenotype that strikingly resembles that of the *plp1⁺* disruptant. On the other hand, it is not clear whether *Drosophila polo* has the equivalent function to that of *S. pombe plo1⁺* in septation. A septation defect in the fission yeast might be equivalent to a defect in cytokinesis in higher eukaryotes. Polyploid cells can be seen in larval brains, together with multipolar spindles in male meiosis. It is, however, difficult to tell whether these phenotypes are the consequence of defects in cytokinesis or mitosis (Sunkel and Glover 1988). It will be worthwhile to reexamine these aspects of the *polo* phenotype in the light of the findings in this paper.

The general relationship between the spindle formation and function and cytokinesis is poorly understood in animal cells. It is known that the position of the centrosomes defines the future position of the cleavage furrow, roughly in the region occupied by the metaphase plate. In this respect, the mechanism differs from that which determines the position of the actin ring and, subsequently, the septum in *S. pombe*. It is nevertheless possible that in fission yeast and in higher eukaryotes, these events are normally triggered at a common stage in the progression through mitosis. The identification of genes from *Drosophila*, fission yeast, and budding yeast that show interactions with *polo*, *plp1⁺*, and *CDC5*, respectively, should prove to be a productive way to elaborate any such conserved mechanism that controls these aspects of mitotic progression.

Materials and methods

S. pombe culture and strains

Culture and manipulation of *S. pombe* were carried out as described by Moreno et al. (1991) or Alfa et al. (1993). All *S. pombe* strains were originally derived from 972h⁻, 975h⁺ and 968h⁹⁰. *cdc2-33*, *cdc25-22*, *cdc10-V50*, and *cdc13* disruptants were described in Nurse et al. (1976), Nasmyth and Nurse (1981), Marks et al. (1992), and Hayles et al. (1994), respectively. *leu1-32*, which is complemented by *S. cerevisiae LEU2*, was crossed into strains for use as a selection marker as necessary. Transformation of *S. pombe* was performed by the high-efficiency lithium method (Okazaki et al. 1991).

Cells were cultured in either rich YES medium or synthetic EMM2 medium containing 100 µg/ml to 250 µg/ml of supplements as necessary. To suppress the *nmt1*⁺ promoter, thiamine was added to EMM2 plus supplements to a final concentration of 4 mM.

Isolation, sequencing, and mapping of the *plol*⁺ gene

A segment of the *plol*⁺ gene was isolated by polymerase chain reaction (PCR) using two primers [oHN20, GCGGAAT-TCAA[AG]AT[ACT]GG[AGCT]GA[CT]; oHN21, [GC][AT][AG]-TA[AG]TC[AGCT]ACCCA[CT]TT] and genomic DNA derived from *S. pombe* 972h⁻. Twelve amplified DNA fragments were cloned in pBluescript (Stratagene) and sequenced. Three of them were identical except for a few bases and show similarity to *polo* homologs. The others were different from each other and neither shows similarity to *polo* nor even contains ORFs. An entire *plol*⁺ gene not containing any DNA derived from PCR was cloned by combination of library screening, integration/rescue, and, finally, gap repair (pHN178). This genomic fragment has an ability to rescue the lethality and cytological abnormality of the disruptant.

The chromosomal position of *plol*⁺ was determined by the hybridization of the *Hind*III 1.9-kb genomic fragment-containing carboxy-terminal part of *plol*⁺ to the set of ordered cosmids and P1 phages used for the *S. pombe* genome project (Hoheisel et al. 1993). The DNA fragment containing the *plol*⁺ gene hybridizes to the phages (including 20e12) derived from one region of the left arm of the first chromosome. No genes have been located previously on the clones.

DNA sequencing was performed by the dideoxy method (Sanger et al. 1977) using T7 DNA polymerase (Pharmacia). Clones for sequencing were made by using a nested deletion kit (Pharmacia).

Plasmid construction

Standard DNA manipulation was carried out according to Sambrook et al. (1989).

All plasmids have the β-lactamase gene as a selection marker in *E. coli*. *S. cerevisiae* *LEU2* was used as a marker to complement *S. pombe* *leu1-32*. *ARS1* or 2 µm of DNA derived from pDB248 (Beach and Nurse 1981) was used as an *ARS* (autonomous replicating sequence) for all plasmids propagated in *S. pombe*.

To express *plol*⁺ under the control of the conditional *nmt1*⁺ promoter, pREP1, pREP41, and pREP81 were used. pREP41 and pREP81 have a modified promoter with expression levels that are 10 and 100 times less than that of pREP1 (Basi et al. 1993; Maundrell 1993). A *Nde*I (CATATG) site was created at the second ATG (the real initiation codon) in the ORF using PCR. The PCR fragment was sequenced to confirm that no base substitutions were introduced. The 2.8-kb *Nde*I–*Scal* fragment was connected into *Nde*I–*Sma*I sites of pREP1, pREP41, and pREP81 to create pHN204, pHN205, and pHN206, respectively. pHN197, pHN198, and pHN200 were constructed to attempt to express *plol*⁺ from the first ATG by subcloning the 3-kb *Hind*III–*Scal* fragment into the *Bam*HI–*Sma*I sites of pREP1, pREP41, and pREP81, respectively, using a *Bam*HI–*Hind*III linker. pHN199 was constructed by connecting the 1.9-kb *Hind*III fragment to pREP1 via the pBluescript polylinker to overproduce the carboxy-terminal half (360 residues).

pHN178 and pHN191 are the *ARS* plasmids containing the entire *plol*⁺ genomic DNA. pHN178 contains the 7-kb *Xba*I–*Hind*III genomic fragment. This plasmid was finally constructed by gap repair using flanking DNA fragments of *plol*⁺

gene. pHN191 has a 3.2-kb *Bgl*II–*Hind*III genomic fragment containing *plol*⁺, *ARS1*, and *S. cerevisiae* *LEU2*. pHN191 was used to introduce the *plol*⁺ gene in *S. pombe*.

pHN184 was constructed for expressing the carboxy-terminal region of *plol*⁺ in *E. coli*. A 2.0-kb *Hind*III fragment containing the carboxy-terminal 360 amino acids was subcloned into pET-23a (Novagen) to produce a 381-amino-acid fusion protein under the control of the T7 RNA polymerase promoter.

Construction of the *plol* disruptant

A 2.2-kb *Hpa*I–*Bgl*II fragment containing the promoter and the coding sequence, except the carboxy-terminal 21 residues, was replaced by a *Hind*III 1.8-kb fragment of *ura4*⁺ with a multi-linker of pBluescript (Stratagene). A 3.4-kb fragment containing the *plol*⁺ gene disrupted by the *ura4*⁺ gene was purified and used for transformation of a diploid (13X16; *h*⁺/*h*⁻ *leu1-32/leu1-32 ura4-d18/ura4-d18 ade6-210/ade6-216*). Stable *Ura*⁺ diploid transformants were selected. We selected transformants that had the desired replacement determined by genomic Southern hybridization. Genomic Southern blots probed with the 1.9-kb *Hind*III fragment containing the carboxy-terminal region of *plol*⁺ identified a 12-kb *Eco*RV band and a 7.5-kb *Bgl*II band in the disruptant diploid, in addition to an 18-kb *Eco*RV band and 5.3-kb and 2.5-kb *Bgl*II bands also observed in wild type. Genomic Southern hybridization probed with a 0.4-kb *Hind*III–*Bgl*II fragment containing the 5'-flanking region of *plol*⁺ gave a 5.3-kb *Eco*RV band and a 7.5-kb *Bgl*II band in the disruptant diploid in addition to 18-kb *Eco*RV and 2.5-kb *Bgl*II bands also observed in wild type.

Cytological methods

DAPI staining was carried out using a variation of the procedure described by Toda et al. (1981). Cells were fixed for 10 min to 1 hr on ice by the addition of glutaraldehyde (BDH) to a final concentration of 2.5%. After washing three times in ice-cold distilled water, the fixed cells were resuspended in ~50 µl of ice-cold water and kept on ice for a short time. One microliter of cell suspension was mixed with 1 µl of 20 µg/ml DAPI (4,6-diamidino-2-phenylindole; Sigma) on a glass slide. Stained cells were immediately observed by fluorescence microscopy under UV light excitation.

Rhodamine–phalloidin (Sigma) staining was carried out according to Marks and Hyams (1985). Immunostaining was carried out as described in Hagan and Hyams (1988). Microtubules and SPBs were stained by anti-α-tubulin mouse monoclonal antibody TAT1 (Woods et al. 1989) and anti-sad1⁺ rabbit polyclonal antibody (I. Hagan and M. Yanagida, in prep.), respectively. These are visualized by second antibodies, FITC-conjugated anti-mouse IgG (Jackson) and Cy3-conjugated anti-rabbit antibody (Sigma). Fluorescence microscopy was carried out using Nikon Microphot and Zeiss Axiophot microscopes with appropriate 100× and 63× objectives and optical filters. Data were recorded using TMAX400 (Kodak) and Pan 100 (Kodak) film for photography.

To examine the septum or septal material, cells were stained with Calcofluor (final 1 mg/ml; Sigma) or Calcofluor/DAPI (0.2 mg/ml; 20 µg/ml) according to Mitchison and Nurse (1985).

Cell concentration in cultures was determined by using a hemocytometer.

DNA content of individual cells was determined by flow cytometry using FACScan (Beckton-Dickinson) after DNA was stained with propidium iodide according to Alfa et al. (1993).

Cytological examination of *plo1* disruptant

For spore germination, the disruptant diploid, in which one copy of *plo1*⁺ was replaced by *ura4*⁺, was sporulated on EMM2 plates containing leucine. After digestion of the ascus wall and the remaining cells by helicase (Industrie Biologique), spores were inoculated into EMM2 medium containing leucine and adenine at 30°C to allow only disruptants to grow. The diploid that carries pHR191 (*plo1*⁺) was used as a control. Spores from the diploid were inoculated into EMM2 containing adenine to allow only disruptants with pHR191 to grow. Only 4% of cells show abnormalities at 15 or 17 hr in the control culture compared with 30% in the culture without the plasmid, indicating that loss of the *plo1*⁺ gene is responsible for the abnormal phenotype.

For the switch-off experiment, the disruptant diploid that has pHN206 (*nmt1-plo1*⁺ on pREP81) was sporulated and disruptants were selected that had pHN206 by culture on EMM2 plates containing adenine. This strain was grown on the EMM2 plate containing adenine. After preculture in EMM2 containing adenine, the culture was diluted in EMM2 containing adenine and thiamine to repress *plo1*⁺ expression. Without the addition of thiamine, no obvious abnormality can be observed.

Overexpression of *plo1*⁺

pHN204 (*nmt1-plo1*⁺ on pREP1) was introduced into various strains to overexpress *plo1*⁺. Strains were maintained on EMM2 plates containing appropriate supplements and thiamine to suppress *plo1*⁺ expression.

Cells were inoculated into EMM2 medium containing thiamine from an EMM2 + thiamine plate. For induction of *plo1*⁺ transcription, cells were washed three times in water and diluted into EMM2 only or into EMM2 containing thiamine as a control. The cell concentration was kept below 5 × 10⁶/ml. Aliquots of the culture were taken at regular intervals for DAPI staining, immunostaining, determination of cell concentration, p34^{cdc2} kinase assay, or flow cytometry.

cdc25-22 and *cdc2-33* strains carrying pHN204 were first cultured at the permissive temperature, 25°C, in the presence of thiamine. Cultures were shifted up to the restrictive temperature, 36°C, 14 hr after withdrawal of thiamine. For *cdc10-V50*, the same procedures were used except shift-up was carried out 12 hours after thiamine withdrawal. The timings of the shift-up were determined by trial and error to induce the overexpression phenotype after complete arrest, and yet to avoid prolonged incubation that leads to leakage from the arrest. Too early an shift-up gives septum induction after a very long arrest, whereas shift-up too late gives septum induction in the cells that have not arrested.

To avoid prolonged arrest, small (G₂) cells were enriched from an asynchronous culture of *cdc25-22* carrying pHN204 following 13 or 15 hr of culture without thiamine at 25°C by two successive steps of centrifugation through 10%–40% lactose gradient. The fractionated small cells were inoculated immediately into EMM2 prewarmed at 36°C.

For the *cdc13* disruptant, the heterozygous diploid (*cdc13::ura4*⁺/+ *leu1-32/leu1-32* *ura4-d18/ura4-d18* *ade6-210/ade6-216* *h*⁺/*h*[−]; Hagan et al. 1988; Hayles et al. 1994) carrying pHN204 was sporulated on EMM2 containing adenine and thiamine. After cells were digested with helicase, spores were inoculated into EMM2 medium containing adenine or both adenine and thiamine as a control.

Protein and immunological methods

Standard immunological methods were used as described in Harlow and Lane (1988).

To express the carboxy-terminal 360 residues of *plo1*⁺, pHN184 was introduced into *E. coli* BL21(DE3) (Studier and Moffat 1986). A protein with the predicted molecular mass (44-kD) was produced only when T7 RNA polymerase is induced by addition of 1 mM Isopropylthiogalactoside (IPTG). Inclusion bodies containing the fusion protein were purified according to Watt et al. (1985), and the protein was purified further using preparative SDS-PAGE. Immunization was carried out by injecting the purified protein with Freund's adjuvants subcutaneously into a rabbit. Sera were collected at 1-month intervals 1 week after each boost. Affinity purification was carried out using the fusion protein according to Smith and Fischer (1984). The quality of antibodies was tested by immunoblotting against *E. coli* total protein expressing the fusion protein and *S. pombe* total crude protein from the strains with single or multicopy *plo1*⁺. Antisera from the fifth and sixth bleeds were used for further experiments.

Total protein from *S. pombe* was prepared for SDS-PAGE as described in Hirano et al. (1988). Immunoblotting detection was done either with the ECL kit (Amersham) or chloronaphthol/H₂O₂ (Sigma) after incubating with rabbit anti-*plo1*⁺ antibody followed by anti-rabbit IgG conjugated with horseradish peroxidase (Jackson).

H1 kinase activity of p34^{cdc2} was measured according to Moreno et al. (1989).

Identification of the *plo1*⁺ gene product

To identify the *plo1*⁺ product, total protein from the strains (*h leu1-32*) containing various ARS plasmids was analyzed by immunoblotting with anti-*plo1*⁺ antibody after SDS-PAGE. Wild type and the strain carrying only the vector give a 77-kD band. Affinity-purified antibody gave the same result. The strain carrying pHN191 (containing *plo1*⁺) gave a much stronger 77-kD band and a weak 49-kD band (this latter band is probably a degradation product as its intensity is variable). This indicates that the 77-kD band is the *plo1*⁺ product. The strain with pHN178 (containing *plo1*⁺ and 4.5 kb of 5'-flanking sequence) gave the same strong 77-kD band. This excludes the possibility of the existence of an upstream exon. To determine the amino terminus of the protein, the strains carrying pHN197 and pHN204 were analyzed. The inducible *nmt1*⁺ promoter connected to the second ATG of the ORF on pHN204 would be expected to produce a protein of the predicted molecular mass 79 kD. When connected upstream of the first ATG of the ORF on pHN197, the predicted molecular mass of the product is 85 kD. The strain with pHN204 gave a strong 77-kD band, the same size as the *plo1*⁺ product, only when induced. The strain with pHN197 gave strong 77-kD bands under both induced and repressive conditions, whereas strong 83-kD and 54-kD bands and weak 41-kD bands were detected only when induced. These data indicate that the second ATG is the true amino terminus. Sometimes, we can detect a very weak band just above the 77-kD band, the identity of which is not clear at this moment.

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Note added in proof

The genomic sequence data of the *plo*⁺ gene have been deposited to the GenBank data library under accession number X85758.

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The conserved *Schizosaccharomyces pombe* kinase plo1, required to form a bipolar spindle, the actin ring, and septum, can drive septum formation in G1 and G2 cells.

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